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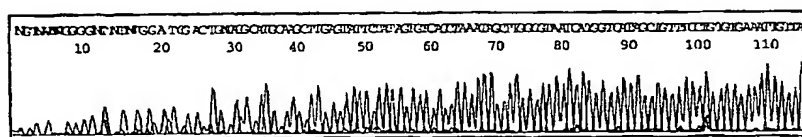
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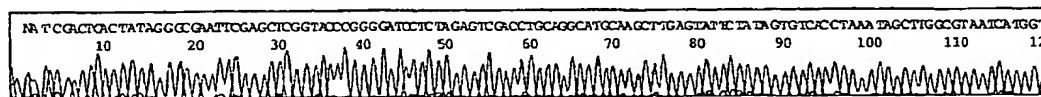
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(54) Title: **PURIFICATION OF DNA SEQUENCING REACTIONS USING SILICA MAGNETIC PARTICLES**

A



B



(57) Abstract: A method and kit are provided for using silica magnetic particles, preferably low porosity silica magnetic particles, to purify DNA extension products from DNA sequencing reactions containing unincorporated dideoxynucleotides and other materials in the solution likely to interfere with sequence analysis, prior to analysis. In a particularly preferred embodiment of the present invention, the method and kit have been designed for use in purifying DNA extension products from DNA sequencing reactions containing unincorporated dideoxynucleotides labeled with fluorescent dyes and other materials in the solution. DNA extension products purified from a sequencing reaction according to the present method or using the present kit produce highly accurate and long reads compared to reactions purified using other methods.

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PURIFICATION OF DNA SEQUENCING REACTIONS USING SILICA MAGNETIC PARTICLES

5

TECHNICAL FIELD

This invention relates generally to methods of purifying DNA extension products from a DNA sequencing reaction containing unincorporated dideoxynucleotides, primers, salts, and other materials which might adversely affect analysis of the DNA extension products of the sequencing reactions. This invention relates, particularly to the use of silica
10 matrices, particularly silica magnetic particles, in purifying DNA extension products from DNA sequencing reactions, including DNA sequencing reactions wherein dideoxynucleotides labeled with fluorescent dyes or primers labeled with fluorescent dyes are used.

BACKGROUND OF THE INVENTION

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In a dideoxy-terminated DNA sequencing reaction, the DNA template to be sequenced, all four deoxynucleotides (i.e., dATP, dCTP, dGTP, and dTTP) or functional equivalents thereof (e.g., dITP or dUTP), a polymerase, a dideoxy nucleotide, and a primer are all present. A dideoxy-terminated DNA sequencing reaction (a sequencing reaction that utilizes dideoxynucleotides to halt DNA extension) is initiated by an oligonucleotide primer
20 hybridizing to a complementary sequence of a strand of the template DNA. The polymerase catalyzes a polymerization reaction in which deoxy- or dideoxynucleotides complementary to the corresponding nucleotides on the template DNA are added to the 3' end of the primer, and then to the 3' end of the growing DNA extension product. The polymerization reaction for any given DNA extension product terminates when a
25 dideoxynucleotide is added to the 3' end of the growing extension product. When the dideoxy-terminated DNA sequencing reaction was first developed, it was designed so that sequencing took place in four different reactions, with a different one of the four dideoxynucleotides in each reaction (i.e. ddATP, ddCTP, ddGTP, and ddTTP). Such reaction products can be detected by using 5' labeled primers or 3' labeled
30 dideoxynucleotides, labeled with a radioactive probe or with a fluorescent label, or by incorporation of a radiolabeled deoxynucleotide (e.g. ³⁵SdATP) or by means that do not require the use of labels. With advancements in labeling and sequencing technology, one

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can now conduct a sequencing reaction in a single container using a mixture of all four dideoxynucleotides, each of which is labeled with a different fluorescent label.

The products of such sequencing reactions can be detected by separating the resulting fragments of DNA by gel electrophoresis or by capillary electrophoresis. In cases
5 where the DNA extension products of the DNA sequencing reaction are not labeled, these products are preferably analyzed by separating the products by gel electrophoresis and staining the resulting gel with a DNA sensitive stain, such as a silver stain. When radioactive labels are used, the DNA extension products can be detected using any suitable means for detection of radioactivity, such as an autoradiogram of an electrophoresis gel.
10 When fluorescent dye labels are used, the DNA extension products can be detected by scanning an electrophoresis gel or capillary array with a fluorescent scanner.

Dideoxy-terminated DNA sequencing reactions, wherein the dideoxynucleotide(s) is labeled tend to have considerably higher background than do reactions wherein the primer is labeled. Such high background interferes with read accuracy, particularly within the first
15 100 bases of the primer. High backgrounds particularly interfere with the reading accuracy of automated fluorescent DNA analysis machines, such as fluorescent gel or capillary electrophoresis analyzers. (e.g., the ABI PRISM[®] 377 DNA Sequencer, LI-COR[®] 4000 or 4200 Sequencer, ALF DNA Sequencer[™], or the ABI PRISM[®] 3700 DNA Analyzer). Unincorporated, labeled dideoxynucleotide(s) in dideoxy-terminated DNA sequencing
20 reaction are primarily responsible for high backgrounds from such reactions, and consequential problems with read accuracy. Unincorporated, labeled primers are responsible for a certain, but lesser amount of background in dideoxy-terminated DNA sequencing reactions wherein labeled primers are used.

Several different methods have been developed for the purification of DNA
25 extension products from a DNA sequencing reaction containing other materials which might affect the accuracy of DNA sequence analysis including, but not limited to, primers, dideoxynucleotides, salt, and polymerase. Such methods include precipitation of the DNA extension product from the reaction solution using ethanol precipitation, liquid chromatography with a gel filtration matrix (see, e.g. Whatman Dye-Terminator Removal
30 Microplate, Technical Applications Manual #5; or DyeEx[™] Dye-Terminator Removal System from QIAGEN), or a filter (e.g., ArrayIt[™] Dye Terminator Clean-Up Kits from TeleChem International, Inc.).

Silica based systems have been developed for use in the purification of plasmid DNA from other material in a solution. Such systems include those which employ controlled pore glass, filters embedded with silica particles, silica gel particles, resins comprising silica in the form of diatomaceous earth, glass fibers or mixtures of the above.

5 Each such silica-based solid phase separation system is configured to reversibly bind nucleic acid materials when placed in contact with a medium containing such materials in the presence of chaotropic agents. The silica-based solid phases are designed to remain bound to the nucleic acid material while the solid phase is exposed to an external force such as centrifugation or vacuum filtration to separate the matrix and nucleic acid material bound

10 thereto from the remaining media components. The nucleic acid material is then eluted from the solid phase by exposing the solid phase to an elution solution, such as water or an elution buffer. Numerous commercial sources offer silica-based resins designed for use in centrifugation and/or filtration isolation systems, e.g., Wizard[®] DNA purification systems products from Promega Corporation (Madison, Wisconsin, U.S.A.), or the QiaPrep[®] DNA

15 isolation systems from Qiagen Corp. (Chatsworth, California, U.S.A.).

Unfortunately, the methods of use of the silica-based solid phases described above and the isolation systems not based upon silica described above all require one to use centrifugation or filtration to perform one or more isolation steps in each method. Such methods are not amenable to automation. The need for automation in DNA sequencing

20 procedures is acute when a large amount of DNA sequencing data is needed, e.g. in sequencing an entire genome.

Magnetically responsive solid phases, such as paramagnetic or superparamagnetic particles, offer an advantage not offered by any of the silica-based solid phases described above. Such particles could be separated from a solution by turning on and off a magnetic

25 force field, by moving a container on to and off of a magnetic separator, or by moving a magnetic separator on to and off of a container. Such activities would be readily adaptable to automation.

Magnetically responsive particles have been developed for use in the isolation of nucleic acids by the direct reversible adsorption of nucleic acids to the particles. See, e.g.,

30 silica gel-based porous particles designed to reversibly bind directly to DNA, such as MagneSil[™] Paramagnetic Particles (Promega), or BioMag[®] Paramagnetic Beads (Polysciences, Warrington, PA, U.S.A.). See also Smith et al., U.S. Patent Number 6,027,945. Magnetically responsive glass beads of a controlled pore size have also been

developed for the isolation of nucleic acids. See, e.g. Magnetic Porous Glass (MPG) particles from CPG, Inc. (Lincoln Park, New Jersey, U.S.A.); or porous magnetic glass particles described in U.S. Pat. Nos. 4,395,271, Beall et al.; 4,233,169, Beall et al.; or 4,297,337, Mansfield et al.

5 Materials and methods are needed which enable one to automate as many steps as possible to quickly and efficiently isolate the products of a DNA sequencing reaction from reagents not used in the reaction, such as unincorporated dye or radioactive labeled dideoxy nucleotides, primers, and salts which can interfere with the analysis of the products of the DNA sequencing reaction. The present invention addresses each of these needs.

10

BRIEF SUMMARY OF THE INVENTION

The present invention provides a method of purifying a DNA extension product from a DNA sequencing reaction prior to analysis, thereby increasing read length and accuracy of sequence analysis. One embodiment of the method comprises: (a) providing
15 DNA sequencing reaction products comprising an unincorporated primer, an unincorporated dideoxynucleotide, and a DNA extension product; (b) combining the DNA sequencing reaction products with silica magnetic particles in an adsorption solution, wherein the DNA extension products selectively adsorb to the particle, thereby forming a complex; and (c) separating the complex from the adsorption solution. The silica magnetic
20 particles used in this embodiment of the invention are preferably macro-porous silica magnetic particles, or more preferably, low porosity silica magnetic particles.

Another embodiment of the method of the present invention is a method of purifying a dye terminal labeled DNA sequencing reaction product, according to the steps comprising: (a) providing dideoxy-terminated DNA sequencing reaction products,
25 comprising an unincorporated dideoxynucleotide labeled with fluorescent dye, and a DNA extension product; (b) combining the DNA sequencing reaction product with silica magnetic particles in an adsorption solution, wherein the DNA extension product selectively adsorbs to the particle, thereby forming a complex; and (c) separating the complex from the adsorption solution.

30 Another embodiment of the invention is a kit for purifying a dideoxy DNA sequencing reaction prior to analysis, comprising: a container comprising silica magnetic particles, a chaotropic agent, and a buffer having a pH of less than about 7.0, preferably a buffer having a pH of 5 or less.

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Applications of the method and uses of the kit of the present invention to purify a variety of different types of DNA sequencing reactions prior to analysis will become apparent from the detailed description of the invention below. Those skilled in the art of this invention will appreciate that the detailed description of the invention is meant to be exemplary only and should not be viewed as limiting the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a printout of sequencing results of the first 115 bases of pGEM[®]-3Zf⁺ DNA template (Promega Cat. # P227) from dye labeled primers each with a sequence identified by SEQ ID NO: 1 using an ABI PRISM 377 DNA Sequencer, after adsorption of the sequencing extension products to MagneSil[™] particles (a type of macroporous silica magnetic particle) in a 0.08M potassium acetate ("KOAc") adsorption solution (pH less than 5.0) and wash of the resulting complex in 70% Ethanol, as described in Example 1.

Figure 1B is a printout of sequencing results of the first 120 bases of pGEM[®]-3Zf⁺ DNA template (Promega Cat. # P227) from dye labeled primers each with a sequence identified by SEQ ID NO: 1 using an ABI PRISM 377 DNA Sequencer, after ethanol precipitation, as described in Example 1.

Figure 2A is a printout of sequencing results of the first 115 bases of pGEM[®]-3Zf⁺ DNA template (Promega Cat. # P227) from a primer represented by SEQ ID NO: 2 obtained using an ABI PRISM 377 DNA Sequencer and ABI PRISM[®] BigDye[™] Terminator reaction mixes, after adsorption of the sequencing extension products to MagneSil[™] particles in a 0.6M GTC/0.08M KOAc adsorption solution and wash of the resulting complex in 70% Ethanol, as described in Example 2.

Figure 2B is a printout of sequencing results of the first 120 bases of pGEM[®]-3Zf⁺ DNA template (Promega Cat. # P227) from a primer represented by SEQ ID NO: 2 obtained using an ABI PRISM 377 DNA Sequencer and ABI PRISM[®] BigDye[™] Terminator reaction mixes after ethanol precipitation, as described in Example 2.

Figure 3 is a printout of sequencing results of the first 115 bases of pGEM[®]-3Zf⁺ DNA template (Promega Cat. # P227) from a primer represented by SEQ ID NO: 2 obtained using an ABI PRISM 377 DNA Sequencer and ABI PRISM[®] BigDye[™] Terminator reaction mixes, after adsorption of the sequencing extension products to

MagneSil™ particles in a 1M GTC/0.08M KOAc adsorption solution and wash of the resulting complex in 90% Ethanol, as described in Example 3.

Figure 4 is a printout of sequencing results of the first 115 bases of pGEM®-3Zf⁺ DNA template (Promega Cat. #P227) from a primer represented by SEQ ID NO: 2 obtained
5 using an ABI PRISM 377 DNA Sequencer and ABI PRISM® BigDye™ Terminator reaction mixes, after adsorption of the sequencing extension products to low porosity silica magnetic particles in a 0.4M GTC/0.08M KOAc adsorption solution and two washes of the resulting complex in 80% Ethanol, as described in Example 4.

Figure 5A is a printout of sequencing results of the first 115 bases of pGEM®-3Zf⁺ DNA template (Promega Cat. # P227) from a primer represented by SEQ ID NO: 2
10 obtained using an ABI PRISM 377 DNA Sequencer and ABI PRISM® BigDye™ Terminator reaction mixes, after adsorption of the sequencing extension products to low porosity silica magnetic particles in a 4.35M GTC/0.03M sodium citrate, pH 4.0 adsorption solution, and after wash of the resulting complex in 90% Ethanol, as described in
15 Example 5.

Figure 5B is a printout of sequencing results of the first 120 bases of pGEM®-3Zf⁺ DNA template (Promega Cat. # P227) from a primer represented by SEQ ID NO: 2
obtained using an ABI PRISM 377 DNA Sequencer and ABI PRISM® BigDye™ Terminator reaction mixes after ethanol precipitation, as described in Example 5.

20

DETAILED DESCRIPTION OF THE INVENTION

To facilitate understanding of the invention, a number of terms are defined below.

The term "chaotropic agent" as used herein refers to salts of particular ions which, when present in a sufficiently high concentration in an aqueous solution, cause proteins
25 present therein to unfold and nucleic acids to lose secondary structure. It is thought that chaotropic ions have these effects because they disrupt hydrogen-bonding networks that exist in liquid water and thereby make denatured proteins and nucleic acids thermodynamically more stable than their correctly folded or structured counterparts. Chaotropic ions include guanidinium, iodide, perchlorate, and trichloroacetate. Chaotropic
30 agents include guanidine hydrochloride, guanidine thiocyanate (which is sometimes referred to as guanidine isothiocyanate), sodium iodide, sodium perchlorate, and sodium trichloroacetate.

The term "magnetic" as used to refer to silica magnetic particles includes materials which are paramagnetic or superparamagnetic materials. The term "magnetic", as used herein, also encompasses temporarily magnetic materials, such as ferrimagnetic or ferrimagnetic materials. Except where indicated otherwise below, the silica magnetic
5 particles used in this invention preferably comprise a superparamagnetic core coated with siliceous oxide, having a hydrous siliceous oxide adsorptive surface (i.e. a surface characterized by the presence of silanol groups).

The term "nucleic acid" as used herein refers to any DNA or RNA molecule or a DNA/RNA hybrid molecule. The term includes plasmid DNA, DNA or RNA fragments,
10 total RNA, mRNA, genomic DNA, and chromosomal DNA.

The term "solid phase" is used herein in a standard chromatographic sense, to refer to an insoluble, usually rigid, matrix or stationary phase which interacts with a solute, in this case a DNA extension product in a DNA sequencing reaction. The term solid phase, as used herein, specifically includes stationary phases in liquid chromatography (LC), high
15 pressure liquid chromatography (HPLC), particulate matrices embedded into or bound to filters, and magnetic or non-magnetic porous matrix particles which interact with solutes when added directly to a solute mixture.

The term "silica gel" as used herein refers to chromatography grade silica gel, a substance which is commercially available from a number of different sources. Silica gel is
20 most commonly prepared by acidifying a solution containing silicate, e.g. by acidifying sodium silicate to a pH of less than 11, and then allowing the acidified solution to gel. See, e.g. silica preparation discussion in Kurt-Othmer Encyclopedia of Chemical Technology, Vol. 21, 4th ed., Mary Howe-Grant, ed., John Wiley & Sons, pub., 1997, p. 1021.

As used herein, the term "silica magnetic particles" refers to silica based solid
25 phases which are further comprised of materials which have no magnetic field but which form a magnetic dipole when exposed to a magnetic field, i.e., materials capable of being magnetized in the presence of a magnetic field but which are not themselves magnetic in the absence of such a field.

The term "macroporous silica magnetic particles", as used herein refers to silica
30 magnetic particles with a median particle size of 1 to 10 μm and a surface area, as measured by nitrogen BET method, of at least about 10 m^2/g of particle mass.

The term "low porosity silica magnetic particles", as used herein refers to silica magnetic particles with a median particle size of about 10 μ m to about 20 μ m and a surface area, as measured by nitrogen BET method, of less than about 10 m²/g of particle mass.

DNA molecules are said to have "5' ends" and "3' ends" because mononucleotides are reacted to make oligonucleotides or polynucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage. Therefore, an end of an oligonucleotides or of a polynucleotide, referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide or polynucleotide, also may be said to have 5' and 3' ends.

As used herein, the terms "complementary" or "complementarity" are used herein in reference to polynucleotides (*i.e.*, a sequence of nucleotides) related by the base-pairing rules. Specifically, adenosine ("A") bases pair with thymidine ("T") bases, and guanosine ("G") bases pair with cytidine ("C") bases on oppositely oriented polynucleotides. For example, the sequence "5'-AGT-3'," is complementary to the sequence "3'-TCA-5'."

As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (*i.e.*, the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementarity between the nucleic acids, stringency of the conditions involved, the melting temperature of the formed hybrid, and the G:C ratio within the nucleic acids. An oligonucleotide primer can be hybridized to a DNA template to initiate a DNA sequencing reaction or to amplify DNA, e.g., in a polymerase chain reaction.

The term "oligonucleotide" as used herein is a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than five, and usually ten or more. The exact size will depend on many factors, which in turn depends on the ultimate function or use of the oligonucleotide. The oligonucleotide may be generated in any manner, including, but not limited to, chemical synthesis, DNA replication, reverse transcription, or a combination thereof.

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis

of a primer extension product which is complementary to a nucleic acid strand is induced, (*i.e.*, in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligonucleotide, more preferably an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

As used herein, the term "purified" or "to purify" means a process or the result of any process which removes some contaminants from the component of interest, such as a DNA extension product. The percent of a purified component is thereby increased in the sample.

In the method of the present invention, a DNA extension product is purified from a dideoxy DNA sequencing reaction prior to analysis of the DNA extension product, using silica magnetic particles. In the purification process, the sequencing DNA extension products are selectively adsorbed to the particles in an adsorption solution, thereby forming a complex, preferably leaving material in solution that might interfere with the analysis of the sequencing extension products (e.g. fluorescently-labeled dideoxy-terminators or primers). The complex is subsequently separated from the adsorption solution. The kit of the present invention provides silica magnetic particles and an adsorption solution designed for use in practicing the method of the present invention.

The method and kit of the present invention are suitable for use in purifying a DNA extension product from a dideoxy-terminated DNA sequencing reaction wherein either the primer or the dideoxy-nucleotide(s) has been labeled with either a radioactive label (e.g., P^{32} or S^{35}) or with a fluorescent dye, or when a radiolabeled nucleotide is incorporated internally in the sequence. The label is preferably a fluorescent dye, preferably a fluorescent dye with a fluorescein donor dye linked to a rhodamine acceptor dye, such as FAM, JOE, TAMARA, or ROX. The label is more preferably a fluorescent dye with a fluorescein donor dye linked to a dichlororhodamine acceptor dye, even more preferably, a fluorescent dye selected from the group consisting of: dichloro[R6G], dichloro[ROX], dichloro[R110], dichloro[TAMRA]. The fluorescein/dichlororhodamine dyes attached to deoxynucleotides or dideoxynucleotides available from ABI under the brand name Big

Dye® deoxynucleotides and BigDye® terminators respectively, are preferred over the fluorescent rhodamine dyes listed above because of their narrower and brighter emission spectra, giving less spectral overlap, and less noise. (See *Automated DNA Sequencing Chemistry Guide*, pub. by PE Applied Biosystems, 1998, pp. 2-2 to 2-6).

5 In addition to isolating sequencing extension products from unincorporated primers or dideoxy nucleotides, the present method also separates the extension products from salts and other components of the extension reaction, which can have a deleterious effect on the analysis of the extension products.

10 When the method is used to purify a DNA extension product from a dideoxy-terminated sequencing reaction in which the primer is labeled, the DNA extension products are preferably adsorbed to the silica magnetic particles, leaving the labeled primers in solution.

The silica magnetic particle used in the method and kit of the present invention can be macro-porous or of low porosity. The silica magnetic particle is most preferably of low
15 porosity. Macro-porous particles tend to be smaller than low porosity particles. However, due to the porosity of the macro-porous particles, the total surface area of a macro-porous particle is greater (on a per weight unit basis) than one would find on larger low porosity silica magnetic particles. Smaller particles are limited in the amount of magnetic material which can be incorporated into such particles compared to larger particles. Thus, even
20 though the surface area of macro-porous particles is greater, low porosity particles can incorporate a greater amount of magnetic material, making them easier to separate from a solution in the presence of a magnetic field.

The median particle size of macro-porous silica magnetic particles used in the present invention is preferably about 1 to 10 μm , more preferably about 3 to 10 μm , and
25 most preferably about 4 to 7 μm . The median particle size of low porosity particles is preferably about 10 to 20 μm , more preferably about 12 to 18 μm , and most preferably about 13 to about 16 μm . The particle size distribution may also be varied. However, a relatively narrow monodal particle size distribution is preferred. The monodal particle size distribution is preferably such that about 80% by weight of the particles are within two
30 standard deviations of the median particle size, more preferably within one standard deviation of the median particle size.

The siliceous oxide coating of each of the macro-porous silica magnetic particles used in the present invention has a total pore volume, as measured by nitrogen BET method,

of at least about 0.2 ml/g, more preferably about 0.5 to 1.5 ml/g based on the total mass of the particles. Of the total pore volume measured by nitrogen BET, preferably at least about 50% is contained in pores having a diameter of 600 Å or greater, more preferably at least about 60%, most preferably about 70 to 85%. The total surface area of a macro-porous silica magnetic particle, as measured by nitrogen BET method, is preferably at least about 10 m²/g, more preferably at least 20 m²/g, even more preferably at least 35 m²/g of particle mass, most preferably about 40 m²/g. The total surface area of a macro-porous silica magnetic particle is preferably no more than about 500 m²/g, more preferably no more than about 100 m²/g, even more preferably no more than about 80 m²/g.

The total surface area of a low porosity silica magnetic particle, as measured by nitrogen BET method, is preferably less than about 10 m²/g, more preferably less than about 5 m²/g, and more preferably less than about 2 m²/g, and even more preferably less than about 0.05 m²/g.

Silica magnetic particles can contain substances, such as transition metals or volatile organics, which could adversely affect the utility of nucleic acids isolated using the particles, when the nucleic acids become substantially contaminated with such substances. Specifically, such contaminants could adversely affect analysis of the sequencing extension products, after purification using the particles, according to the method of the present invention. Any such substances present in the silica magnetic particles used in the method and kit of the present invention are preferably present in a form that does not readily leach out of the particle and into the purified DNA sequencing reaction produced according to the method or using the kit of the present invention. Iron is one such potential contaminant.

Iron, in the form of magnetite, is present at the core of particularly preferred forms of silica magnetic particles used in the method and kit of the present invention. Iron has a broad absorption peak between 260 and 270 nanometers ("nm"). Nucleic acids, such as DNA, have a peak absorption at about 260 nm, so iron contamination in a nucleic acid sample can adversely affect the accuracy of the results of quantitative spectrophotometric analysis of such samples. Any iron containing silica magnetic particles used to isolate DNA sequencing extension products using the present invention preferably do not produce isolated extension products sufficiently contaminated with iron for the iron to interfere with spectrophotometric analysis of the material at or around 260 nm.

The most preferred macro-porous silica magnetic particles used as in the methods and kits of the present invention are siliceous oxide coated particles produced as described in international patent application publication number WO 98/31461. The macro-porous silica magnetic particle production method disclosed therein can also be modified and adapted for use in producing the low porosity silica magnetic particles used in the present invention. Low porosity silica magnetic particles suitable for use in the present invention are commercially available from WR Grace (Catalog No. MP-85). The description of the silica magnetic particles contained in WO 98/31461 is incorporated by reference herein. Regardless of whether macro-porous or low porosity particles are used, the preferred silica magnetic particles leach no more than 50 ppm, more preferably no more than 10 ppm, more preferably no more than 7 ppm, and most preferably no more than 5 ppm of transition metals when assayed as described immediately below.

Leaching of transition metals from the particles is assayed as follows: 0.33 g of the particles (oven dried @110°C) are combined with 20 ml. of 1N HCl aqueous solution (using deionized water). The resulting mixture is then agitated only to disperse the particles. After about 15 minutes total contact time, a portion of the liquid from the mixture is then analyzed for metals content. Any conventional elemental analysis technique may be employed to quantify the amount of transition metal in the resulting liquid, but inductively coupled plasma spectroscopy (ICP) is preferred. Macro-porous silica magnetic particles that meet the above-cited specification for particularly preferred particles are sold under the brand name MagneSil™ Paramagnetic Particles (Promega Corporation). Low porosity silica magnetic particles that meet the above-cited specification are available from WR Grace (Catalog No. MP-85).

For methods of adsorption and desorption of nucleic acids to silica magnetic particles in general, some of which methods are suitable for use in the present invention, see international patent application number PCT/US98/01149 for METHODS OF ISOLATING BIOLOGICAL TARGET MATERIALS USING SILICA MAGNETIC PARTICLES, published as WO 98/31840, incorporated by reference herein. Adsorption of the DNA extension products to the silica magnetic particles used in the present invention preferably takes place in the presence of an adsorption solution.

The adsorption solution used in the method and included in the kit of the present invention is configured to promote selective adsorption of DNA extension products to silica magnetic particles, when combined with the products of a dideoxy DNA sequencing

reaction. The adsorption solution preferably comprises a chaotropic agent and a buffer having a pH of less than about 7.0, preferably having a pH of less than about 6.0, more preferably having a pH of less than about 5.0. The buffer is preferably an acetate buffer or a citrate buffer, more preferably a citrate buffer, most preferably sodium citrate. The
5 chaotropic agent is preferably, sodium iodide, potassium iodide, urea, sodium perchlorate, or a guanidine salt, more preferably guanidine hydrochloride or guanidine thiocyanate.

Once a complex of the silica magnetic particle and DNA extension products has formed in the adsorption solution, any one of a number of different means can be used to separate the complex from the adsorption solution. Suitable separation means include, but
10 are not limited to, vacuum or gravity filtration, decantation, centrifugation, or magnetic force. The complex is most preferably separated from the adsorption solution in the presence of a magnetic force. Any source of magnetic force sufficiently strong to separate the silica magnetic particles from a solution would be suitable for use in separating the complex from the adsorption solution and from other solutions used in preferred additional
15 steps of the present method. However, the magnetic force is preferably provided in the form of a magnetic separation stand, such as one of the MagneSphere[®] Technology Magnetic Separation Stands (Cat. Nos. Z5331 to 3, or Z5341 to 3) from Promega Corporation.

The method of the present invention preferably further comprises a step of washing
20 the complex prior to elution of the DNA extension products therefrom. The composition of any wash solution used to wash the complex is selected to ensure the DNA extension products remain part of the complex. The wash solution preferably comprises at least 50% of a low molecular weight alcohol, such as ethanol or isopropanol, more preferably at least 60% ethanol, even more preferably at least 70% ethanol, even more preferably at least 90%
25 ethanol.

The nucleic acid adsorption solution and any wash solution are preferably prepared from or consist of distilled or deionized water. The distilled, deionized, or other type of water used in the nucleic acid adsorption solution is preferably filtered prior to use, using a filtration that achieves at least 18 mega Ohms-resistance of water. The Nanopure[®]
30 Filtration System (Barnstead) can be used to produce such water. The distilled, deionized, or filtered water can be autoclaved prior to use in the method or in the kit of the present invention.

Elution of a nucleic acid, such as a DNA extension product, from the complex is carried out in the presence of an elution solution selected for its capacity to ensure the release of the extension product from the complex. The elution solution preferably comprises a component selected from the group consisting of water, formamide, and a tracking dye. When elution solution comprises a tracking dye it is preferably a dye suitable for use in tracking the DNA extension products as they are fractionated by gel or capillary electrophoresis. The elution solution is most preferably a loading solution, containing all the components necessary for loading a sample of the DNA extension products onto either an electrophoresis gel or a capillary electrophoresis capillary. The elution solution preferably has a pH of at least about 5.0 and up to about 8.0, more preferably at least about 6.0 and up to about 8.0.

Once the DNA sequencing reaction has been purified, as described above, the DNA extension products are preferably analyzed by gel or capillary electrophoresis. The purified reaction produces results with low backgrounds and high accuracy of read, even close to the primer. Read is considered, herein, to begin at the point closest to a primer in which four bases in a row are accurately read. Thereafter, read tends to be accurate. Fluorescent dye labeled DNA extension products isolated according to the present invention produce at least 98% of readable sequence when a sequence is read out to 600 bases, with readable sequence beginning within 50 bases after the primer sequence, more preferably producing at least 99% of readable sequence out to 600 bases, with readable sequence beginning within 25 bases, even more preferably with readable sequence beginning within 10 bases after the primer sequence.

The following, non-limiting examples teach various embodiments of the invention. In the examples, and elsewhere in the specification and claims, volumes and concentrations are at room temperature unless specified otherwise. The macro-porous magnetic silica particles used in the examples below were all MagneSil™ particles having the general preferred dimensions and siliceous oxide coating described herein. The MagneSil™ particles used in the Examples below were taken from either of two batches of particles having the following characteristics: (1) a BET surface area of 55 m²/g, pore volume of 0.181 ml/g for particles of <600 Å diameter, pore volume of 0.163 ml/g for particles of >600 Å diameter, median particle size of 5.3µm, and iron leach of 2.8 ppm when assayed as described herein above using ICP; or (2) a BET surface area of 49 m²/g, pore volume of

0.160 ml/g (<600 Å diameter), pore volume of 0.163 ml/g (>600 Å diameter), median particle size of 5.5µm, and iron leach of 2.0 ppm.

Five different batches of low porosity particles, having the characteristics set forth in Table 1, below were combined and used in Examples 4 and 5, below.

5

TABLE 1

<u>Batch</u>	<u>Particle Size (µ)</u>	<u>BET (m²/gm)</u>	<u>Fe₂O₃(wt%)</u>	<u>SiO₂(wt%)</u>	<u>Leach Resistance</u>
1	13.1	2	14.6	84.5	<0.8
2	13.7	1	15.3	82.1	<0.8
3	15.6	not available	14.0	84.8	<1.0
10 4	13.7	not available	14.7	84.9	<1.0
5	14.2	not available	14.5	84.8	<1.0

One skilled in the art of the present invention will be able to use the teachings of the present disclosure to select matrices suitable for use in the method and in the kit of the present invention other than the specific embodiment of the silanized silica magnetic particles produced as described in Example 3, below, and used in the following Examples. Specifically, the Examples should not be construed as limiting the scope of the present invention. Other types of silica magnetic particles, and methods of using the same to purify DNA sequencing reactions according to the present invention will be apparent to those skilled in the art of chromatographic separations and molecular biology, in view of the present disclosure.

EXAMPLES

The following examples are given to illustrate various aspects of the invention, without limiting the scope thereof:

EXAMPLE 1: Purification of a Dye Primer Sequencing Reaction

pGEM[®]-3Zf+ DNA template (Promega Cat. #P227) was sequenced, using a Dye Primer DNA sequencing kit from ABI (Cat. # 402112) and the cycling conditions listed in Table 2 below the Wizard[®] DNA Purification products (*i.e.*, Promega, Cat. #A7100).

Four (4) microtubes were set up for each sample (an A, G, C, and T tube, respectively), with the samples indicated in Table 2, below. The contents of each microtube were mixed in a pipette tip.

TABLE 2

	<u>A Tube</u>	<u>C Tube</u>	<u>G Tube</u>	<u>T Tube</u>
DNA Sample	1.0 μ l	1.0 μ l	2.0 μ l	2.0 μ l
A mix	4.0 μ l			
C mix		4.0 μ l		
G mix			8.0 μ l	
T mix				8.0 μ l

The set of tubes prepared as described above was then cycled in a 9600 Thermocycler,
 5 according to the cycling protocol shown in Table 3, below:

TABLE 3

1 cycle	96.0°C	2.0 min
25 cycles	96.0°C	10.0 sec.
	55.0°C	5.0 sec.
	70.0°C	1.0 min.
1 cycle	96.0°C	10.0 sec.
	70.0°C	1.0 min.
Hold	4.0°C	soak

The thermocycler was heated prior to use. The samples were not placed into the
 10 thermocycler until the block temperature had reached 85° C. The entire thermocycling
 program took about 1 hour and 15 minutes.

The sequencing reaction was divided into twelve tubes (30 μ l each) and treated in
 12 different ways to remove the dye primers prior to running the sequence on an ABI
 PRISM® 377 DNA Sequencer.

15 The reactions in tubes one and two were ethanol ("EtOH") precipitated by
 combining with 80 μ l chilled 95% ethanol and incubating on ice for 15 minutes. The tubes
 were then centrifuged at 14,000 rpm in a microcentrifuge for 30 minutes at 4°C. The
 supernatant was removed and the DNA pellet washed with 100 μ l chilled 70% ethanol. The
 tubes were again centrifuged at 14,000 rpm in a microcentrifuge for 5 min at 4°C. The

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supernatant was removed, the pellet dried and resuspended in 6 μ l loading dye (5 parts deionized formamide: 1 part EDTA/Blue Dextran).

The reactions in tubes 3-12 were treated with MagneSil™ particles in various binding buffers to remove the dye primers. For each reaction, MagneSil™ particles (45 μ l) were equilibrated in 600 μ l of an adsorption solution consisting of the concentration of guanidine thiocyanate ("GTC") given in Table 4, below, and 0.08 M potassium acetate ("KOAc"), pH 4.8, and washed three times in 600 μ l of the adsorption solution.

10

TABLE 4

Sample	Binding Buffer	Ethanol Wash
1	(EtOH precipitation)	70%
2	(EtOH precipitation)	70%
3	0.0 M GTC / 0.08 M KOAc	70%
4	0.2 M GTC / 0.08 M KOAc	70%
5	0.4 M GTC / 0.08 M KOAc	70%
6	0.6 M GTC / 0.08 M KOAc	70%
7	0.8 M GTC / 0.08 M KOAc	70%
8	0.0 M GTC / 0.08 M KOAc	80%
9	0.2 M GTC / 0.08 M KOAc	80%
10	0.4 M GTC / 0.08 M KOAc	80%
11	0.6 M GTC / 0.08 M KOAc	80%
12	0.8 M GTC / 0.08 M KOAc	80%

Samples 3-12 were processed as follows, prior to analysis: 200 μ l of each of the MagneSil™ particles in binding buffer was added to an aliquot of the sequencing reaction, allowed to bind for one minute, magnetized, and the supernatant removed. The pellets of MagneSil™ particles for samples 3-7 were washed twice in 70% ethanol, while the pellets for samples 8-12 were washed twice in 80% ethanol. After the final wash was removed the pellets were allowed to air dry for 5 minutes and each resuspended in 50 μ l water. Then 200 μ l of the appropriate binding buffer, as used initially with the particles, was added to each pellet, the pellet magnetized, and the supernatant removed. The pellets were washed

with ethanol as described above.

The pellets were air dried for 10 minutes. Then 20 µl water was added to the pellet to elute the DNA from the pellet. The DNA solution was vacuum dried for 20 minutes and resuspended in 6 µl loading dye. (All samples were analyzed on an ABI 377 by loading 1.5ul of each sample and electrophoresing for approx. 10hrs.)

Table 5, below, provides a summary of the results of analyzing the samples purified, as described above, on an ABI 377 DNA Sequencer. The percent accuracy results in Table 5 are percent accuracy in 600 bases read. The final column of data in Table 5 shows the number of bases from the primer at which readable sequence began.

TABLE 5

Sample	Binding Buffer	Ethanol Wash	600 base % Accuracy	Read from Primer
1	(EtOH precipitation)	70%	93.3%	12
2	0.0 M GTC / 0.08 M KOAc	70%	85.6%	107
3	0.2 M GTC / 0.08 M KOAc	70%	76.1%	90
4	0.4 M GTC / 0.08 M KOAc	70%	99.8%	70
5	0.6 M GTC / 0.08 M KOAc	70%	78.8%	108
6	0.8 M GTC / 0.08 M KOAc	70%	88.8%	71
7	0.0 M GTC / 0.08 M KOAc	80%	82.5%	99
8	0.2 M GTC / 0.08 M KOAc	80%	98.5%	59
9	0.4 M GTC / 0.08 M KOAc	80%	98.8%	101
10	0.6 M GTC / 0.08 M KOAc	80%	98.8%	121
11	0.8 M GTC / 0.08 M KOAc	80%	77.3%	124

As indicated in Table 5, above, results from this experiment indicated that various GTC and EtOH concentrations gave different overall sequence quality. This reflected the overall binding, washing, and elution efficiencies with the method. In all of the samples, the small fragments were being lost, cutting the read length short. However, once the sequence started, several of the conditions gave excellent accuracy results. The 0.4M GTC with 70% EtOH washes and the 0.6M GTC with 80% EtOH washes gave the best overall results- intense signal strength, good accuracy once the sequence started.

The first 115 bases of the sequence from sample 3 are shown in Figure 1A. This

sequence is representative of typical sequencing results obtained by purifying the dye primer labeled sequencing reactions using MagneSil™ particles under the conditions described herein above. The sequence using sample purified with the ethanol precipitation method is shown in Figure 1B.

5

Example 2: Comparison of Ethanol Precipitation to Use of MagneSil™ Particles in Purifying a Dye Terminator Labeled Sequencing Reaction.

This example reflects the sequencing reaction using BigDye™ Terminator Sequencing conditions with wash conditions using either ethanol precipitation or
10 MagneSil™ particles (Promega, part #) in 0.6 M GTC/ 0.08 M KOAc adsorption solution as described in Example 1.

pGEM®-3Zf⁺ DNA template (Promega, P227) was sequenced using a primer having the sequence identified by SEQ ID NO: 2 (5' GTTTTCCCAGTCACGAC 3') and the ABI PRISM® BigDye™ Terminator Sequencing Chemistry (Perkin Elmer Catalog No.
15 4303149) A 7X master mix of BigDye™ Terminator sequencing reaction was assembled by combining 56 µl Terminator Ready Reaction Mix (dideoxy-A labeled with dichloro [R6G], dideoxy-C labeled with dichloro [R0X], dideoxy-G labeled with dichloro [R110], dideoxy-T labeled with dichloro[TAMRA], a mixture of dATP, dCTP, dITP, and dUTP, AmpliTaq DNA Polymerase FS, thermally stable pyrophosphatase, MgCl₂, and Tris buffer, pH 9.0)
20 17.5 µl of template DNA (200 ng/µl), 12.6 µl Primer 1 (10 µg/µl), and 53.9 µl water.

The sequencing reactions were assembled with 1/7th of the master mix and performed as instructed in the BigDye™ Terminator kit (ABI Catalog No. 4303152). The amplification was performed in a 9600 Thermocycler with the cycling profile described in Table 6, below:

25

TABLE 6

Step #1 (repeat for 25 cycles)	96.0°C 50.0°C 60.0°C	10.0 sec. 5.0 sec. 4.0 min.
Step #2	4.0°C	hold

30

The resulting sequencing reactions were then either ethanol precipitated or treated with MagneSil™ particles to remove the BigDye Terminators. The ethanol precipitation was performed on samples 1 and 2 by adding 16 µl water and 64 µl room temperature
95% ethanol to the sequencing reaction. The tubes were incubated at room temperature

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for 15 minutes and then centrifuged at 14,000 rpm in a microcentrifuge for 20 min at 4°C. The supernatant was removed and the DNA pellet washed with 250 µl room temperature 70% ethanol. The tubes were then centrifuged at 14,000 rpm in a microcentrifuge for 10 min at 4°C. The supernatant was removed and the pellet was vacuum dried for 15 minutes. Each dried pellet was resuspended in 6 µl loading dye.

Samples 3-6 were purified using MagneSil™ particles, as follows. First, 37.5 µl MagneSil™ particles were mixed with 1 ml binding buffer (0.6 M GTC / 0.08 M KOAc) as described in example 1. Then 200 µl of the MagneSil™ particles in the binding buffer were added to each sample and allowed to bind for 5 minutes at room temperature. The tubes were gently mixed to keep the particles in solution. The particles were magnetized and the supernatant removed. The particle pellets were washed once with 200 µl 80% ethanol and remagnetized. The supernatants were removed and the pellets air-dried for 10 minutes. Then 10 µl loading dye was added to each pellet to elute the DNA, the tubes were incubated at room temperature for 1 minute and then magnetized.

All samples were analyzed on an ABI 377 by loading 1.5 µl of each sample and electrophoresing for about 10 hours. In all of the samples, the small fragments (i.e., within 50 bases of the primer) were not readable. All the samples had an accuracy of readable sequence of about 90% when read out to 610 bases.

The sequence of the first 120 bases of one of the samples purified with the MagneSil™ particles in this example, a sample representative of all samples, is shown in Figure 2A. The sequence using sample purified with the ethanol precipitation method is shown in Figure 2B.

Example 3: MagneSil™ Particles in Modified BigDye™ Terminator Purification

In this example, BigDye™ Terminators were removed from a sequencing reaction with conditions much like those described in Example 2 for MagneSil™ Particles. The difference illustrated in this example is that the adsorption solution was 1 M GTC, 80 mM KOAc at pH 4.8 and the wash solution was 90% ethanol. The BigDye™ Terminator chemistry was performed as recommended by the manufacturer with the exception that a 1:4 dilution of the terminator ready reaction mix was used. See above example for the formulation of this mix

The readable sequence in the reaction solution purified with the MagneSil™

particles, as described herein, began 31 bases after the primer sequence and there was 100% accuracy in the sequence when read out to 600 bases. However, the dye removal was poor as illustrated in Figure 3 which shows uninterpretable sequence at the beginning of the sequence.

5

Example 4: Effect of Dilution of Reaction Mix on Sequencing After Removing BigDye™ Terminators by Three Different Methods

This example compared three methods for removal of BigDye™ Terminators from sequencing reactions. The methods used were 1) ethanol precipitation, 2) MagneSil™ particles, and 3) Low-porosity magnetic particles.

10

The sequencing reactions were performed with the ABI PRISM® BigDye™ Terminator Sequencing System according to manufacturer's instruction with the template and primer described above in Example 2. The termination ready mix of the sequencing system was either not diluted, 1:4 diluted, or 1:8 diluted to represent conditions often utilized by end-users of this system. The amplification conditions were previously described in Example 2.

15

The sequencing reactions (20 µl) purified by ethanol precipitation were each combined with 16 µl nanopure water and 64 µl room temperature 95% ethanol. The reactions were incubated at room temperature for 15 minutes and then centrifuged at 14,000 rpm in a microcentrifuge for 20 minutes at 4°C. The supernatants were removed and the pellets washed with 250 µl room temperature 70% ethanol. The tubes were centrifuged at 14,000 rpm in a microcentrifuge for 10 minutes at 4°C. The supernatants were removed and the pellets vacuum-dried for 15 minutes. The pellets were resuspended in 10 µl loading dye and 1.5 µl run on an ABI 377 Sequencer as described in Example 2.

20

Magnetic particles were prepared by mixing 45 µl MagneSil™ particles (porous) in 1080 µl binding buffer and by mixing 45 µl Low-porosity magnetic particles in 1080 µl binding buffer. The binding buffer was 0.6 M GTC / 0.08 M KOAc for both types of particles. The porous particles were 100 mg/ml, the low-porosity particles were 133 mg/ml. Then 180 µl of the particles/binding buffer mixture was added to the appropriate reactions as listed below. They were allowed to bind for 5 minutes with frequent mixing of the tubes. The tubes were then placed on a magnet, the particles allowed to collect against the tube and the supernatants removed. The pellets were washed once with 200 µl 80% ethanol and the

25

30

supernatants removed. The pellets were allowed to air dry and the nucleic acid eluted in 10 μ l loading dye for 1 minute, magnetized, and 1.5 μ l of the supernatant run on an ABI 377 Sequencer as described in Example 2. The samples were loaded and run on the sequencer as described in Table 7, below, producing the results shown in the final columns of the table:

TABLE 7

<u>Sample</u>	<u>Termination Mix dilution</u>	<u>Purification method</u>	<u>600base % Accuracy</u>	<u>Read from Primer</u>
1	no dilution	Ethanol precipitation	99.5	26
2	no dilution	Ethanol precipitation	99.5	52
3	no dilution	Porous particles	96.3	60
4	no dilution	Porous particles	96.3	68
5	no dilution	Low-porosity particles	96.0	68
6	no dilution	Low-porosity particles	96.2	69
7	1:4	Ethanol precipitation	99.2	17
8	1:4	Ethanol precipitation	99.7	21
9	1:4	Porous particles	98.2	36
10	1:4	Porous particles	97.2	39
11	1:4	Low-porosity particles	98.7	25
12	1:4	Low-porosity particles	99.0	26
13	1:8	Ethanol precipitation	97.3	18
14	1:8	Ethanol precipitation	99.8	21
15	1:8	Porous particles	not done	Not done
16	1:8	Porous particles	99.7	25
17	1:8	Low-porosity particles	98.8	26
18	1:8	Low-porosity particles	98.5	26

A scan of the first 120 bases of the sequence from sample 11, above, are shown in Figure 4. This reaction had 98.8% accuracy of readable sequence when read out to 600 bases, but the readable sequence did not begin until 45 bases after the primer sequence. Dye removal was poor with these conditions. The dilution of the termination mix clearly helped bring the readable sequence closer to the primer. Undiluted termination mix did not yield readable sequence until 50-75 bases away from the primer. The ethanol precipitated

sample was able to be read from about 25 bases away from the primer sequence.

EXAMPLE 5: Use of Low-Porosity Silica Magnetic Particles for BigDye™ Terminator Purification

5 Four different lots of low-porosity silica magnetic particles in a citrate buffer were tested for effectiveness of removing BigDye™ terminators from sequencing reactions and compared to an ethanol precipitation method, as described below. (Other experiments conducted indicate that there is no DNA ladder binding to the particles without the citrate or acetate present).

10 The five batches of low porosity particles described in Table 1, above, were combined and divided into four lots, A, B, C, and D, that were tested in triplicate to remove the terminators. Twenty microliters of sequencing reaction was added to the following mixture:

	Low-porosity magnetic particles	10 µl
15	7 M GHCl	143 µl
	0.5 M NaCitrate, pH 4.0	16 µl
	water	41 µl

The tubes were incubated at room temperature for 5 minutes with periodic mixing. The particles were magnetized and the supernatant was removed. The particles were then
20 washed twice, 5 minutes each wash, with 100 µl 90% ethanol. The particles were then magnetized and the supernatant removed. The particles were allowed to air dry for 15 minutes and then resuspended in loading dye and run on and ABI 377 sequencer.

Alternatively, the sequencing reaction was purified by ethanol precipitation prior to running on the sequencer. To a 20 µl sample was added 16 µl water and 64 µl 95% ethanol.
25 The tube was incubated at room temperature for 15 minutes and then centrifuged at 11,000 rpm in a microcentrifuge for 20 minutes at 4°C. The supernatant was removed and the pellet washed in 70% ethanol. The pellet was dried in a vacuum and resuspended in loading dye.

The results are shown in Table 8, below:

30

TABLE 8

Sample	Purification Treatment	Read from primer	600 bases accuracy	700 bases accuracy
1	Lot A	9	99.2%	98.4%
2	Lot A	10	99.3%	99.3%
3	Lot A	19	99.7%	99.4%
4	Lot B	16	99.5%	98.1%
5	Lot B	10	99.2%	98.7%
6	Lot B	10	99.3%	99.3%
7	Lot C	10	99.5%	99.1%
8	Lot C	5	99.3%	98.9%
9	Lot C	5	99.2%	98.7%
10	Lot D	10	99.2%	99.1%
11	Lot D	5	99.7%	98.6%
12	Lot D	10	99.7%	99.4%
13	ethanol ppt	8	99.0%	99.0%
14	ethanol ppt	18	99.5%	98.9%

The electropherogram for the first 120 bases of Sample 7 sequence above is shown in Figure 5. Overall, this method provided excellent removal of the BigDye™ terminators, an excellent read start typically of 5 to 10 bases from the primer and a read accuracy greater than 99% out to 600 bases from the primer.

The sequence produced using these conditions (citrate instead of acetate) is the best of all the conditions tested in the Examples, above, as shown in Figure 5A. Sequence could be read beginning from 5-15 bases away from the primer with between 98.1 to 99.4% accuracy out to 700 bases, with all lots tested and all replicates isolated using the low-porosity particles, as described above. The sequence using sample purified with the ethanol precipitation method is shown in Figure 5B.

CLAIMS

What is claimed is:

1. A method of purifying DNA extension products from a DNA sequencing reaction prior to analysis, comprising:
 - 5 a) providing DNA sequencing reaction products, comprising unincorporated dideoxynucleotides, unincorporated primers, a salt, and DNA extension products;
 - b) combining the DNA sequencing reaction products with silica magnetic particles in an adsorption solution, wherein the DNA extension products selectively
10 adsorb to the particle, thereby forming a complex; and
 - c) separating the complex from the adsorption solution.
2. The method of claim 1, wherein the primer is labeled.
- 15 3. The method of claim 2, wherein the dideoxynucleotide is labeled.
4. The method of claim 3, wherein the dideoxynucleotide is labeled with a fluorescent dye selected from the group consisting of dichloro[R6G], dichloro[ROX], dichloro[R110],
20 dichloro[TAMRA].
5. The method of claim 1, wherein the silica magnetic particles are macro-porous silica magnetic particles, having a median particle size of about 1 μm to about 10 μm , and a nitrogen BET surface area of at least about 10 m^2/g .
- 25 6. The method of claim 1, wherein the silica magnetic particles are low porosity silica magnetic particles, having a median particle size of about 10 μm to about 20 μm and a nitrogen BET surface area of less than about 10 m^2/g .
7. The method of claim 1, wherein the adsorption solution comprises a chaotropic
30 agent and a buffer with a pH of less than about 7.0.
8. The method of claim 7, wherein chaotropic agent is selected from the group consisting of guanidine thiocyanate and guanidine hydrochloride.

9. The method of claim 7, wherein the buffer is potassium acetate.
10. The method of claim 7 wherein the buffer is sodium citrate.
- 5 11. The method of claim 1, wherein the complex is separated from the adsorption solution in the presence of a magnetic field.
- 10 12. The method of claim 1, further comprising washing the complex by combining the complex with a wash solution, mixing the complex and wash solution, and separating the complex from the wash solution.
- 15 13. The method of claim 12, wherein the wash solution comprises at least 60% of a low molecular weight alcohol selected from the group consisting of isopropanol and ethanol.
- 20 14. The method of claim 1, further comprising eluting the DNA sequencing products from the complex by combining the complex with an elution solution having a pH of at least about 5.0 and up to about 8.0, thereby promoting the desorption of the DNA sequencing products from the complex into the elution solution.
- 25 15. The method of claim 14, wherein the elution solution comprises a component selected from the group consisting of water, formamide, and a tracking dye.
- 30 16. A method of purifying DNA extension products from a dideoxy DNA sequencing reaction prior to analysis, according to the steps comprising:
- a) providing dideoxy DNA sequencing reaction products, comprising unincorporated dideoxy-nucleotide labeled with fluorescent dye, unincorporated primer, and DNA extension products;
 - b) combining the DNA sequencing reaction products with silica magnetic particles in an adsorption solution, wherein the DNA extension products selectively adsorb to the particle, thereby forming a complex; and
 - c) separating the complex from the adsorption solution.

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17. The method of claim 16, wherein the fluorescent dye is selected from the group consisting of dichloro[R6G], dichloro[ROX], dichloro[R110], dichloro[TAMRA].

5 18. The method of claim 16, wherein each of the silica magnetic particles comprises silica gel with a siliceous oxide coating, and a paramagnetic core.

10 19. The method of claim 16, wherein the silica magnetic particles are macro-porous silica magnetic particles, having a median particle size of about 1 μm to about 10 μm , and a total nitrogen BET surface area of at least about 10 m^2/g .

20. The method of claim 16, wherein the silica magnetic particles are low porosity silica magnetic particles, having a median particle size of about 10 μm to about 20 μm and a nitrogen BET surface area of less than about 10 m^2/g .

15 21. The method of claim 16, wherein the adsorption solution comprises a chaotropic agent and a buffer having a pH of up to about 5.0.

20 22. The method of claim 21, wherein chaotropic agent is selected from the group consisting of guanidine thiocyanate and guanidine hydrochloride.

23. The method of claim 21, wherein the buffer is potassium acetate.

24. The method of claim 21, wherein the buffer is sodium citrate.

25 25. The method of claim 16, wherein the complex is separated from the adsorption solution in the presence of a magnetic field.

30 26. The method of claim 16, further comprising washing the complex by combining the complex with a wash solution, mixing the complex and wash solution, and separating the complex from the wash solution.

27. The method of claim 26, wherein the wash solution comprises at least 60% of a low molecular weight alcohol selected from the group consisting of isopropanol and ethanol.

28. The method of claim 16, further comprising eluting the DNA extension products from the complex prior to analyzing the DNA, by combining the complex with an elution solution with a pH of at least about 5.0 up to about 8.0, thereby promoting the desorption of the DNA sequencing products from the complex into the elution solution.
29. The method of claim 28, wherein the elution comprises a component selected from the group consisting of water, formamide, and a tracking dye.
30. The method of claim 16, further comprising a step of evaluating the DNA extension product by loading the complex directly onto an evaluation apparatus selected from the group consisting of gel electrophoresis and capillary electrophoresis, and separating the DNA from the complex in an electric field created during electrophoresis.
31. A kit for purifying a DNA extension product from a dideoxy DNA sequencing reaction prior to analysis, comprising:
a container comprising silica magnetic particles, a chaotropic agent and a buffer having a pH of less than about 7.0.
32. The kit of claim 31, wherein the silica magnetic particles are macro-porous silica magnetic particles, having a median particle size of about 1 μm to about 10 μm , and a nitrogen BET surface area of at least about 10 m^2/g .
33. The kit of claim 31, wherein the silica magnetic particles are low porosity silica magnetic particles, having a median particle size of up to about 10 μm to about 20 μm and a nitrogen BET surface area of less than about 10 m^2/g .
34. The kit of claim 31, wherein the chaotropic agent is selected from the group consisting of guanidine thiocyanate and guanidine hydrochloride.
35. The kit of claim 31, wherein the concentration of chaotropic agent in the container is at least about 1 molar and up to about 7 molar.

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36. The kit of claim 31, wherein the buffer is sodium citrate.
37. The kit of claim 31, wherein the buffer is potassium acetate.

FIG. 1A

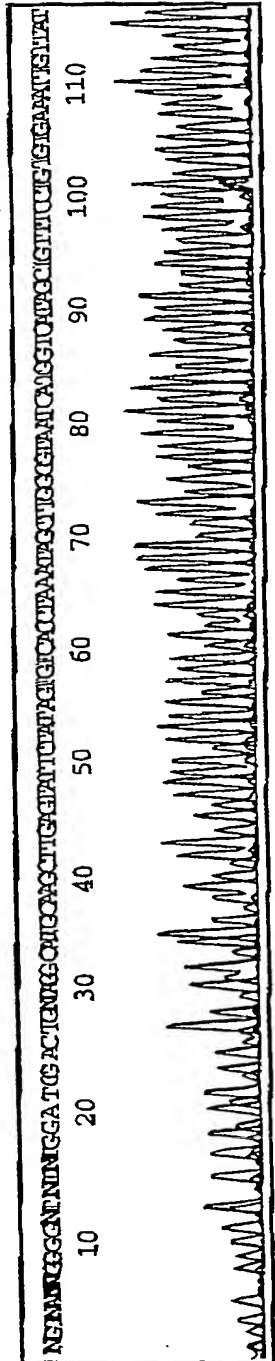
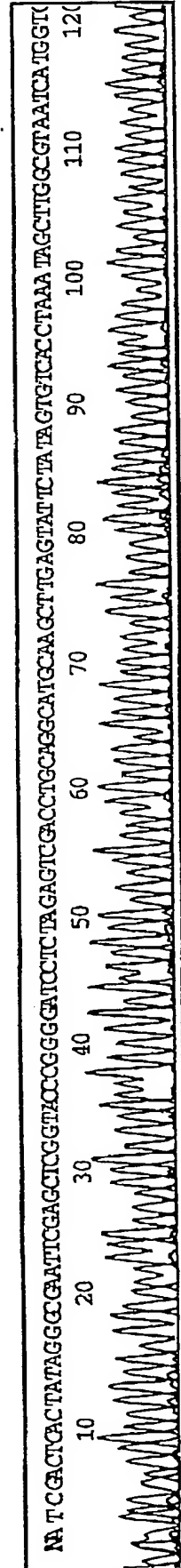
 $\frac{1}{4}$

FIG. 1B



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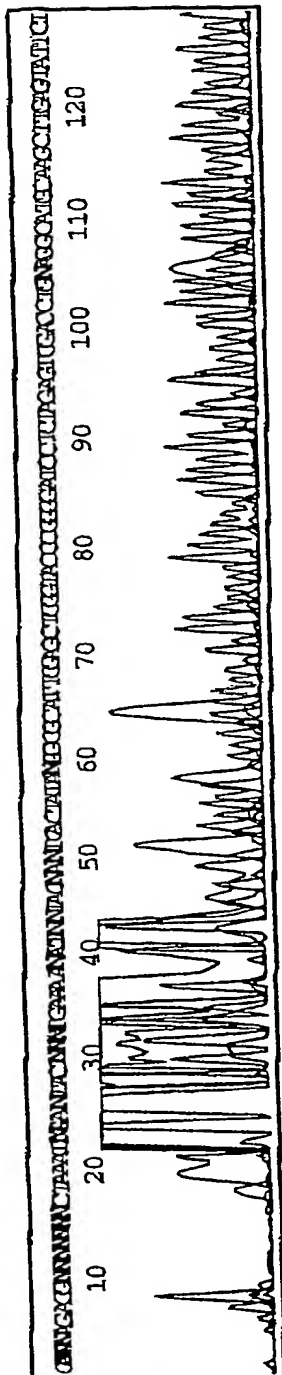
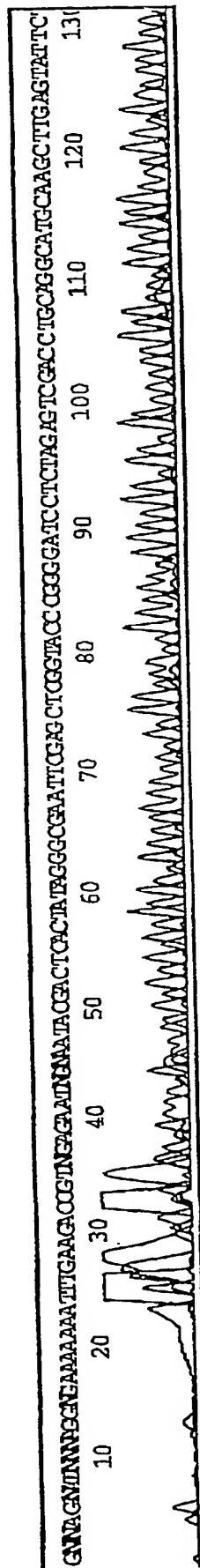


FIG. 2B



Year	Percentage (%)
1960	10
1961	15
1962	20
1963	25
1964	20
1965	25
1966	20
1967	15
1968	10
1969	15
1970	10
1971	15
1972	10
1973	15
1974	10
1975	15
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1977	15
1978	10
1979	15
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1983	15
1984	10
1985	15
1986	10
1987	15
1988	10
1989	15
1990	10

FIG. 5A

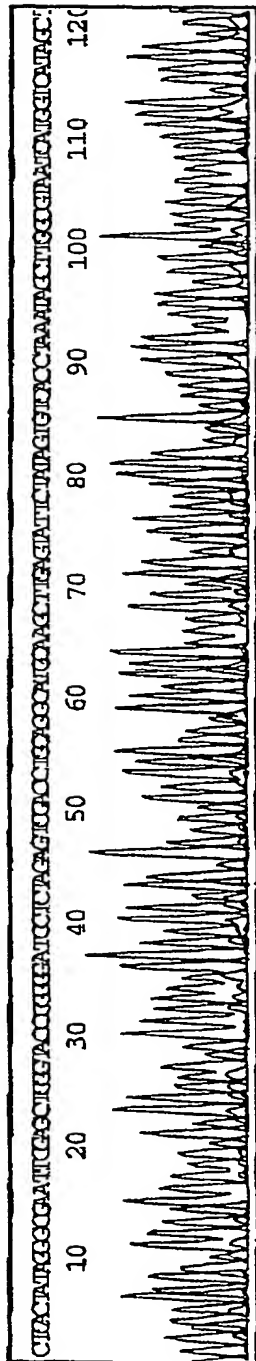
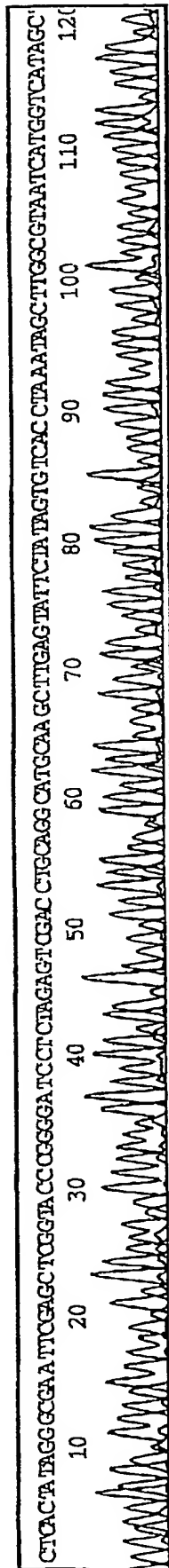


FIG. 5B



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LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
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(54) Title: PURIFICATION OF DNA SEQUENCING REACTIONS USING SILICA MAGNETIC PARTICLES

(57) Abstract: A method and kit are provided for using silica magnetic particles, preferably low porosity silica magnetic particles, to purify DNA extension products from DNA sequencing reactions containing unincorporated dideoxynucleotides and other materials in the solution likely to interfere with sequence analysis, prior to analysis. In a particularly preferred embodiment of the present invention, the method and kit have been designed for use in purifying DNA extension products from DNA sequencing reactions containing unincorporated dideoxynucleotides labeled with fluorescent dyes and other materials in the solution. DNA extension products purified from a sequencing reaction according to the present method or using the present kit produce highly accurate and long reads compared to reactions purified using other methods.

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INTERNATIONAL SEARCH REPORT

International Application No.

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A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 027 945 A (SMITH CRAIG E ET AL) 22 February 2000 (2000-02-22) column 1 -column 6 column 8 -column 9 column 11 -column 15 claims 1-22	1-37
A	US 5 683 875 A (LICHTENWALTER KAY) 4 November 1997 (1997-11-04) column 3 -column 10	1-37
A	US 4 233 169 A (BEALL GEORGE H ET AL) 11 November 1980 (1980-11-11) the whole document	1-37
P, X	US 6 194 562 B1 (CRESWELL DONALD A ET AL) 27 February 2001 (2001-02-27) column 5 -column 10	1-37

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 01/43364

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 6027945	A	22-02-2000	AU 732756 B2	26-04-2001
			AU 6647598 A	07-08-1998
			BR 9805897 A	24-08-1999
			CA 2249393 A1	23-07-1998
			DE 895546 T1	17-10-2002
			EP 0895546 A1	10-02-1999
			JP 3253638 B2	04-02-2002
			JP 11509742 T	31-08-1999
			US 2002086326 A1	04-07-2002
			WO 9831840 A1	23-07-1998
			US 6368800 B1	09-04-2002
US 5683875	A	04-11-1997	NONE	
US 4233169	A	11-11-1980	AU 538041 B2	26-07-1984
			AU 5723180 A	16-10-1980
			DE 3013915 A1	30-10-1980
			FR 2453836 A1	07-11-1980
			GB 2047679 A ,B	03-12-1980
			JP 1485771 C	14-03-1989
			JP 55144445 A	11-11-1980
			JP 63032732 B	01-07-1988
			US 4395271 A	26-07-1983
US 6194562	B1	27-02-2001	AU 740145 B2	01-11-2001
			AU 3651399 A	08-11-1999
			CA 2329067 A1	28-10-1999
			EP 1071695 A1	31-01-2001
			JP 2002512252 T	23-04-2002
			WO 9954340 A1	28-10-1999
			US 6284470 B1	04-09-2001

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